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The overall objective of this project is to provide a detailed description of the organization of the primate, including the human, circadian timing system. The specific objectives for this project period were as follows: 1) Analyze the organization of the human retinohypothalamic tract (RHT); 2) Analyze the organization of the human suprachiasmatic nucleus (SCN); 3) Analyze the organization of the macaque monkey SCN; 4) Analyze the distribution of retinal afferents to the macaque monkey intergeniculate leaflet (IGL) and the organization of its intrinsic neurons; 5) Analyze the organization of the human IGL; 6) Determine whether there is a human homologue of the rodent sexually dimorphic nucleus (SDN-POA).

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AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

FINAL TECHNICAL REPORT

"ORGANIZATION OF THE HUMAN CIRCADIAN SYSTEM"

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Project Period: 6/1/93 - 5/31/96

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TECHNICAL REPORT

Objectives: The overall objective of this project is to provide a detailed description of the organization of the primate, including the human, circadian timing system. The specific objectives for this project period were as follows: 1) Analyze the organization of the human retinohypothalamic tract (RHT); 2) Analyze the organization of the human suprachiasmatic nucleus (SCN); 3) Analyze the organization of the macaque monkey SCN; 4) Analyze the distribution of retinal afferents to the macaque monkey intergeniculate leaflet (IGL) and the organization of its intrinsic neurons; 5) Analyze the organization of the human IGL; 6) Determine whether there is a human homologue of the rodent sexually dimorphic nucleus (SDN-POA).

Research Accomplishments: These will be described for each of the objectives noted above.

1. The Human Retinohypothalamic Tract (RHT). Work by Takatsuji and colleagues (1991), and confirmed by Mikkelsen and Larsen (1993), has shown that there is a dense plexus of substance P-immunoreactive (SP+) fibers in the ventrolateral SCN of the rat that is lost following enucleation. This is interpreted to indicate that there is a subset of SP+ retinal ganglion cells that project in the RHT. As there is no known marker for the RHT in the human, the demonstration of an RHT by immunohistochemistry would provide valuable information. Two prior studies reported evidence for an RHT in the human brain. Sadun et al (1984) used a method for demonstration of degenerated axons and Friedman et al (1991) used DiI to show a retinohypothalamic projection. Both studies reported evidence for optic chiasm axons entering the SCN but no data indicating the density or distribution of the projection. Using antisera against SP, we demonstrated a dense projection of SP+ axons in the ventral SCN in the human brain in a distribution overlapping the location of vasoactive intestinal polypeptide-immunoreactive (VIP+) neurons and axons. The plexus extends in the rostral SCN ventrally into pockets of neuropil in the optic chiasm. Caudally, it is localized in the central SCN over the VIP+ elements and surrounded by vasopressin-immunoreactive (VP+) neurons and fibers. At very caudal levels of the SCN, the SP+ plexus becomes co-extensive with a SP+ plexus that arises in the adjacent anterior hypothalamus (Moore and Speh, 1994). These data appear to demonstrate the location and distribution of the RHT in the human SCN but do not permit conclusions about RHT projections outside the SCN as are observed in other animals (Johnson et al, 1988).

2. Human SCN. In prior studies (Moore, 1992, 1993), we reported observations on the human SCN derived from this research program. Over the last 3 years, it has become evident that both PMI and fixation are important variables in determining the quality of human material. In this time, our local Alzheimer's Disease Research Center (ADRC) has reduced the PMI for both Alzheimer and control brains. In the last application we proposed studying the effect of age on the SCN. With a small, 1 year pilot grant ($15,000) from the ADRC, we collected a series of control and Alzheimer brains. The ADRC funding also permitted some processing of the material from Alzheimer brains. Each of 14 control and 10 Alzheimer brains was dissected to produce a block of tissue extending from the lamina terminalis to the mammillary bodies. The blocks were cut in the coronal plane at 40 μm and every fourth section was stained for neurotensin (NP)-, vasopressin (VP), vasoactive intestinal polypeptide (VIP)- or neuropeptide Y (NPY)-

immunoreactivity using the avidin biotin method. Sections from five brains from the control and Alzheimer's groups were prepared as above but with the fifth section stained with cresyl violet (Nissl stain). The mean SCN neuron number was determined from the Nissl material from each group of brains using an unbiased stereological method. For the control brains, this was 47,307 ± 5215 (mean ± S.D.) and for the Alzheimer's brains, it was 42,534 ± 4255. This 10% difference between Alzheimer and control SCN neuron number is not statistically significant either by a t test or a non-parametric statistic (Mann-Whitney, U = 5, p = .075). We should note that there is one major difficulty in performing cell counts on Nissl material from human SCN. The boundaries of the SCN are not clear and the counts were made using the border of the nucleus from immunohistochemical material stained for NT extrapolated to adjacent Nissl-stained sections. On this basis, we expect the total SCN neuron number derived from these counts to be high, almost certainly significantly higher than the real neuron number. The comparison of Alzheimer and control brains makes it clear that neither age nor Alzheimer's disease affect SCN neuron number.

The data for the immunohistochemical material are shown in Table 1, below. For each immunohistochemically-defined group, neuron number was determined by counting each identifiable neuron in all sections through the nucleus and correcting for the number of sections sampled. No other correction was employed. In comparing these counts with ones obtained from selected brains using an unbiased stereological method, we found only minor differences between the two methods.

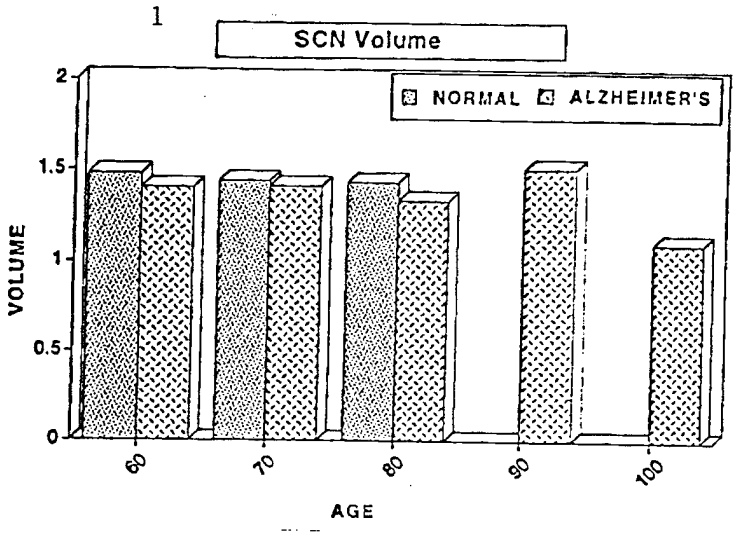
TABLE 1

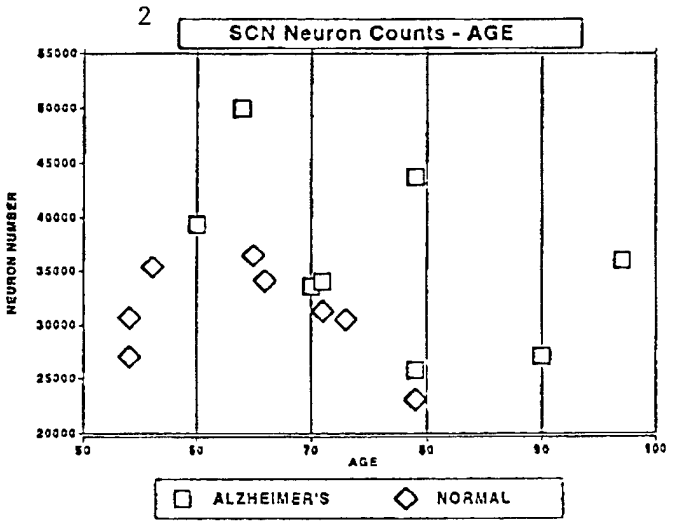
SCN NEURON NUMBER IN IMMUNOHISTOCHEMICALLY IDENTIFIED POPULATIONS IN CONTROL AND ALZHEIMER BRAINS

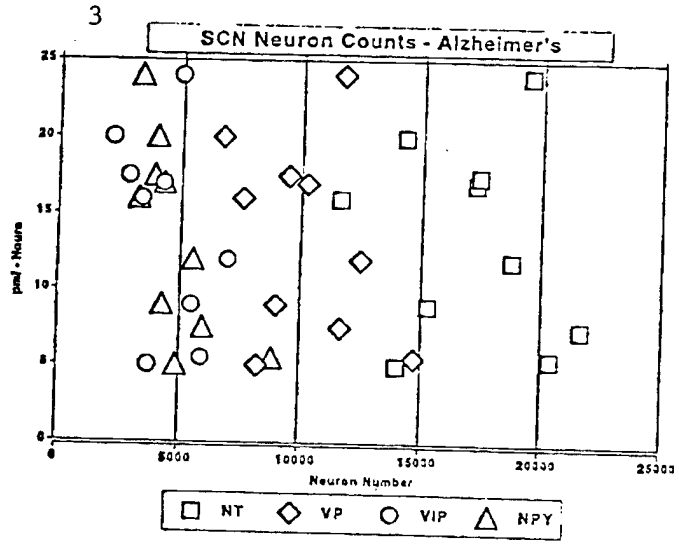
PEPTIDE	Neuron Number		Neuron Area - μm^2		Neuron Diameter - μm	
	Control	Alzheimer	Control	Alzheimer	Control	Alzheimer
NT	16118 ± 2628	17011 ± 3195	119 ± 19	102 ± 15	15.4 ± 1.4	14.1 ± 1.0
VP	8283 ± 2053	10177 ± 2455	117 ± 15	106 ± 16	15.4 ± 1.0	14.6 ± 1.3
NPY	4943 ± 1438	1814 ± 1672	118 ± 17	98 ± 10	15.5 ± 1.4	14.1 ± 1.0
VIP	2988 ± 1008	4404 ± 1560	95 ± 16	83 ± 12	14.0 ± 1.4	12.8 ± 1.2
Total Neuron Number	32073 ± 4772	35597 ±7766	All measurements are presented as mean ± S.D. None of the differences between control and Alzheimer's parameters are significant.			

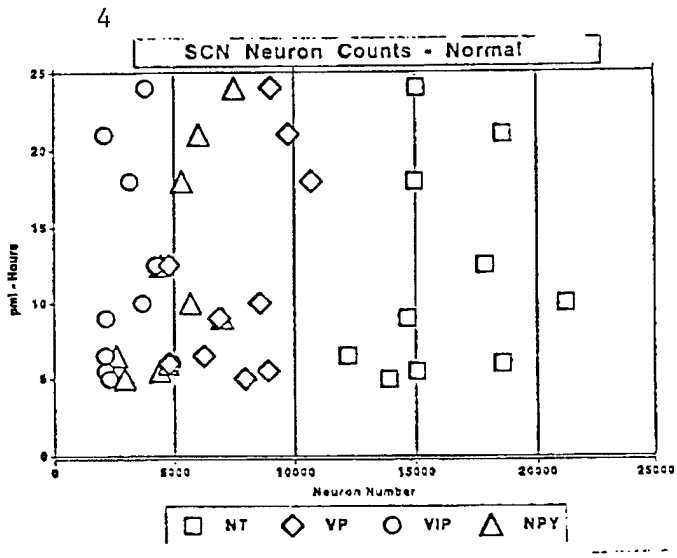
Total SCN neuron number obtained from the immunohistochemical analysis is approximately 25%-30% below the estimates obtained from Nissl stain analysis. As noted above, we believe that the Nissl analysis over-estimates total SCN neuron number because of the method used to define the boundaries of the nucleus. In addition, there may be one, or more, SCN populations that are not identified in this analysis. For example, we have recent data which indicate that the rat SCN contains a population of gastrin releasing peptide (GRP)-containing neurons that is distinct from the VIP+ group.

Similarly, there is evidence that the rat SCN contains a population of angiotensin II (-AII)-containing neurons. At present, the antiserum against GRP that is available to us does not identify neurons in the human brain and we do not have an antiserum against AII. It is our intent to generate oligonucleotide DNA probes against GRP and AII mRNA to determine whether these populations exist in the human brain. In addition to determining neuron number, we have used the MCID M-2 Image Analysis System to measure parameters of neuron size in the SCN, diameter and area. These are shown in Table 1. Both mean diameter and area for each population, NT+, VP+, NPY+ and VIP+ is smaller for Alzheimer brains than for controls. Since this is true for each population, it is almost certainly a reflection of a functional change. The measurements for each of the three major neuron populations, NT+, VP+ and NPY+ are very similar, the neurons have a mean diameter of approximately 15.5 μm and a mean area of approximately 118 μm^2 for controls. The VIP+ neurons are clearly smaller with a diameter of 14.0 μm and an area of 95 μm^2 and represent a different population.









In Figure 1, the volume of the SCN in controls and Alzheimer brains is displayed as a function of age. In this, it is clear that there is no alteration of SCN volume either as a function of age or the presence of Alzheimer's disease. It is noteworthy that the oldest brain in our series, from a 99 year old female, shows an SCN volume that is only slightly less than SCNs from younger ages, both control and Alzheimer's. Total neuron number from this SCN was 36,000 (NT, 17256; VP, 10218; NPY, 4278; VIP, 4248). As might be expected, there is no significant difference between control (4.7 ± 1.4 mm) and Alzheimer's (3.7 ± 1.5 mm) with respect to SCN length. SCN neuron number (Figure 2) does not appear to be affected by age. However, these data may be confused by three brains, Alzheimer's brains at ages 64, 79 and 99 with very high counts. If these were excluded from Figure 2, one could obtain the impression that cell number was decreasing from ages 60-80 or 90. Individual neuron counts for each of the histochemically-identified cell groups are quite stable across the age and are not affected by PMI (Figures 3 and 4).

The evident conclusions from this analysis are as follows: 1) SCN neuron number is not affected by PMI or age; 2) the total SCN number appears to be about 45,000 as assessed from Nissl material (but is likely that this is a 5-10% over-assessment); 3) the four major groups of SCN neurons, those containing NT, VP, NPY and VIP, comprise about 35,000 (75-85% of the total as estimated from Nissl material; 4) NP, VP and NPY neurons appear to comprise a single population on the basis of neuron size but VIP neurons are smaller and likely to be a separate population; 5) there are no significant differences between Alzheimer's SCN and control SCN with respect to total neuron number of any identified population but the size of SCN neurons appears reduced in Alzheimer's disease.

In the rat, the SCN appears to be comprised predominantly of GABA neurons (Moore and Speh, 1993). We would expect this to be the case in the human as well but GABA and GAD antisera do not work in our hands on postmortem material. For that reason, we have initiated an analysis of the human SCN using cDNA probes against GAD message. To provide a background with *in situ* hybridization methods, we initiated the study by preparing probes against rat GAD_{65} and GAD_{67}. These two isoforms of GAD occur, respectively, as a cytoplasmic enzyme, presumably involved in metabolic actions of GABA (GAD_{65}), and as a vesicular enzyme involved in production of GABA as a neurotransmitter (GAD_{67}). This is the current view of Dr. Alan Tobin, one of the major workers in the field, who also believes that these isoforms co-exist in all GABA neurons (A. J. Tobin, personal communication). For this reason, we prepared oligonucleotide (24-45 bases) probes against both GAD_{65} and GAD_{67} message and mixed these for *in situ* hybridization. This probe produced a very robust signal in rat SCN. With combined *in situ* hybridization histochemistry (ISHH) and cresyl violet staining, it appears that essentially all SCN neurons are GAD positive in the rat, as we would expect from the GABA immunohistochemistry (Moore and Speh, 1993). We then constructed probes against human GAD and used these for ISHH analysis. As in the rat, there is a very dense signal over the SCN which, with exposure to emulsion and cresyl violet staining, is over individual neurons and appears to label most, if not all, SCN neurons. At present we have done ISHH for GAD mRNA on 5 human brains using fresh material and this is presented in Gao and Moore (1996).

We have used a number of antisera other than the ones against the peptides noted above including ones against galanin (GAL), calcitonin gene-related peptide (CGRP), tyrosine hydroxylase, dopamine-β-hydroxylase, serotonin and the serotonin-producing enzyme, tryptophan hydroxylase, glial fibrillary acidic protein (GFAP) and enkephalin in immunohistochemical studies of the human SCN. Of the peptides, only CGRP and GAL show a significant staining of axonal plexuses both of which are sparse and lie in the center of the SCN. The source of these plexuses is unknown but they presumably arise from nearby anterior hypothalamic CGRP+ and GAL+ neurons. The absence of serotonin and GFAP staining is noteworthy. There is dense GFAP staining in the rodent SCN (Morin et al, 1989) which shows a circadian fluctuation that has been interpreted to indicate a role for astrocytes in circadian function (Lavialle and Servier, 1993). In contrast, in our human material the SCN shows less GFAP immunoreactivity than surrounding anterior hypothalamus. The significance of this marked interspecies difference is unclear but it seems likely that there is an important role for glia, beyond their normal role in neuronal function, in the generation and regulation of circadian function in the human SCN. The absence of serotonin immunoreactivity is expected as small molecule transmitters do not survive the immediate postmortem period in sufficient amounts to be shown by immunohistochemistry. The serotonin plexus presumably could be shown either by antisera against tryptophan hydroxylase or the serotonin transporter. Finally, we have used two antisera generated against calcium binding proteins, calretinin (CAR) and calbindin (CAB), to study the human SCN. In the rat, we have found a large population of CAR+ neurons in the dorsolateral portion of the nucleus. We do not identify any CAR+ neurons in the human SCN. It seems likely, though, that this represents a combination of low peptide content and a relatively insensitive antiserum. We have identified a population of CAB+ neurons in the human SCN. These are located predominantly in the optic chiasm and the adjacent ventral SCN. Part of this population corresponds to the VIP+ neurons but the CAB+ group is larger and more extensive. Their localization is such that they clearly receive RHT input, indicating that retinorecipient neurons can be divided into at least two groups, one characterized by VIP content and the other by CAB content.

3. The Monkey SCN. The macaque monkey SCN is similar to SCNs of other mammals with respect to location in the hypothalamus and appearance in Nissl stains. It lies above the optic chiasm, lateral to the third ventricle and intercalated between the large, deeply-stained neurons of the supraoptic nucleus laterally and the paraventricular nucleus dorsally. The monkey SCN is 1.4 ± 0.3 in length with a volume of 0.20 ± 0.04 mm^3 ($\pm$ S.D.; n = 6). We have prepared one animal for analysis of the RHT using cholera toxin, β-subunit, injected into the vitreous of one eye, survival 72 hours, and anterograde transport of the cholera toxin shown by an antiserum to cholera toxin. The cholera toxin produced a chemical ophthalmitis affecting the ciliary process, lens, iris and anterior chamber. There was no inflammation, however, of the retina and transport was moderate. The inflammation was sufficiently severe that we have not repeated the experiment. Although the amount of the cholera toxin transported was not optimal, not as heavy as in rodents, the material has permitted us to determine that the monkey RHT is very similar to that in rodents. The primary labeling is in the central portion of the SCN. This begins very rostrally in the nucleus and extends to its caudal end. There is dense axonal labeling in an area which overlaps the distribution of VIP+ perikarya, as in rodents. This area also includes a number of SP+ perikarya which are situated slightly dorsal to the VIP+ cells. As in the human, there are CAB+ cells and fibers in the ventral SCN. RHT fibers extend into the zone surrounding the VIP+ and SP+ cells, a zone

characterized by VP+ cells. Some fibers also extend beyond the borders of the nucleus into the anterior hypothalamic area lateral and dorsal to the SCN. At caudal levels of the SCN, the RHT plexus extends and becomes less dense. As the SCN disappears caudally, the plexus is quite large and innervates a broad region of retrochiasmatic area extending caudally to a zone at the transition between anterior and tuberal hypothalamus. The major neuron populations in the monkey SCN are the VP+ and VIP+ groups. We have not identified any NPY+ neurons nor any GRP+ or somatostatin-containing cells. The several GABA antisera available to us do not stain any cells in the SCN but there is very dense staining, more dense than in surrounding anterior hypothalamus, with the GAD antiserum. Even with this, however, we do not observe stained perikarya. It is our interpretation that this represents a low content of GAD and GABA in the perikarya, not a lack of GABA-containing perikarya. It is difficult to show GAD or GABA in the rat SCN without colchicine pretreatment which cannot be done in the monkey. We presume that the GABAergic nature of monkey SCN neurons can be shown with ISHH. Morphometry of the identified neuronal population in the monkey SCN is shown in Table 2.

TABLE 2

NEURON NUMBER AND SIZE IN THE MONKEY SCN

PARAMETER MEASURED*	VASOPRESSIN (VP)	VASOACTIVE INTESTINAL POLYPEPTIDE (VIP)	SUBSTANCE P (SP)
Neuron Number	5476 ± 1354	5109 ± 2260	3975 ± 1782
Neuron Diameter	10.5 ± 0.3	10.4 ± 0.5	10.4 ± 0.5
Neuron Area	56 ± 4	55 ± 8	57 ± 3

*All measurements are presented as mean ± S.D. From five brains.

In addition to the axonal plexuses associated with the intrinsic neuronal plexuses, there are two additional prominent axonal plexuses. The first is a dense plexus of serotonin axons and terminals, presumably arising from the midbrain raphe nuclei. As in rodents, this is located in the same region as the main RHT plexus. The second is a dense and similarly located NPY+ plexus, presumably arising from the IGL. There are a few NT+ neurons in the monkey SCN (< 500), many fewer than in the human but widely spread over the nucleus. Thus, the monkey SCN is very similar to that of rodents but differs from the human in having many fewer NT+ neurons and totally lacking NPY+ neurons.

4. The Monkey IGL. In Nissl material, the pregeniculate nucleus (Jones, 1985) is a group of neurons situated dorsal and medial to the dorsal lateral geniculate (DLG) nucleus. It has been conventional to view this as ventral lateral geniculate (VLG) but we have argued previously (Moore, 1989) that at least part of it should be IGL. In the

studies we have conducted, we have analyzed cytoarchitecture, the retinal input to the pregeniculate area and the pattern of NPY-, ENK- and SP-immunoreactivity. The area contains two evident subdivisions in Nissl material. One is located along the medial and dorsal surface of the DLG, is comprised of a homogeneous set of medium-sized, lightly staining neurons which are continuous medially with the zona incerta. The second, dorsal and lateral to the first, contains slightly larger and darker neurons and is continuous with the thalamic reticular nucleus dorsally. In cholera toxin material demonstrating retinal projections, there is a dense contralateral projection to part of this latter area, the remainder and the entire area on the ipsilateral side receives very sparse retinal input. The area immediately adjacent to the DLG receives a bilateral retinal input, approximately twice as dense to the contralateral as to the ipsilateral side. Both areas contain NPY+ neurons and an axonal plexus but these are more dense in the area adjacent to the DLG. Both have SP+ and ENK+ axonal plexuses. Our conclusion is that the area adjacent to DLG represents IGL, largely on the basis of the pattern of retinal projections, its location in the geniculate complex and its content of NPY+ neurons. Among these, the pattern of retinal projections and location are primary considerations; NPY+ neurons are a necessary but not sufficient criterion. The content of ENK+ and SP+ axons did not discriminate. We did not see any ENK+ or SP+ neuronal perikarya. This is similar to the rat where ENK+ neurons are only clearly evident in colchicine-pretreated material and SP-producing neurons can only be shown by ISHH. In this regard, we have prepared recently an oligonucleotide probe for SP mRNA which demonstrates positive neurons in the rat IGL.

5. The Human IGL. As in the monkey, the human pregeniculate nucleus lies dorsal and medial to the DLG. In Nissl material, it does not have evident subdivisions and, absent knowing the pattern of retinal afferents, it is difficult to delineate IGL and VLG. The entire nucleus contains NPY+ neurons and axonal plexus and ENK+ and SP+ axonal plexuses. Recently, we had the opportunity to view material prepared by H. Braak and E. Braak in Frankfurt using a special stain. this clearly delineates VLG and IGL in a manner that appears identical to the monkey.

In the prior application, we proposed five projects. The first was to perform Golgi studies on the human SCN. It has not been possible to begin this work with the press of other studies and this was our lowest priority analysis. The second project was to define the primate SCN and retrochiasmatic area, both monkey and human. We have essentially completed the analysis of the SCN and papers on the human and monkey are in preparation. We have begun the analysis of the retrochiasmatic area. We also have begun an ISHH analysis of the human SCN using probes to GAD mRNA and will initiate similar work in the monkey in the next 6 months. The third project was to analyze SCN projections in the monkey and human using VIP+ material. The material for this is all in hand and the analysis is in progress. We expect to have this completed in the next 6 months. We have accumulated some human material in which double label immunocytochemistry, for VIP and either CRH, VP or oxytocin, is performed to obtain insight into the circadian control of endocrine and autonomic function. This will be expanded and analyzed in the next year. The fourth project was to analyze the human RHT using SP as a marker. This was completed and a paper published (Moore and Speh, 1994). The fifth study was to analyze the effects of aging on the human SCN. The interesting outcome of this is that in contrast to prior reports (Swaab et al, 1985, Goudsmit et al, 1990), we find no change in the SCN with age and only changes in cell size, not cell number, in Alzheimer's disease. It is worth noting that we see no change in

either the length or volume of the SCN in Alzheimer's disease and senile plaques, which are abundant in the adjacent hypothalamus, do not invade the SCN. These data suggest that there is no alteration of either SCN neuron number or SCN neuropil in aging and Alzheimer's disease. We used the Alzheimer brains both for their intrinsic interest and because they represent a major source of aged brains.

6. The Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA). In the rat, there is a component of the medial preoptic area which is sexually dimorphic, larger and containing more neurons in males than females. In recent studies, we have found that the rat SDN-POA consists of a population of GABA-containing neurons. This was established using *in situ* hybridization histochemistry with oligonucleotide probes to the message for the GABA-forming enzyme, glutamic acid decarboxylase (GAD). GAD comes in two isoforms, GAD_{65} and GAD_{67}. All neurons in the rodent SDN-POA appear to contain both GAD_{65} and GAD_{67}. In the human hypothalamus, there is a small nucleus dorsal and lateral to the SCN which is believed to be the homologue of the rat SDN-POA. Using fresh human hypothalamus, we find that this nucleus also is made up of neurons that express GAD_{65} and GAD_{67} (Gao and Moore, 1996). This is further evidence that the rodent and human nuclei are homologous.

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6. Moore, R.Y. The human suprachiasmatic nucleus: Control and Alzheimer's disease. In preparation.

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